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Original Article

Serological diagnosis of liver fibrosis by quantitative estimation of serum biomarkers using Luminex xMAP assay

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Abstract: Background: Conventional serological markers of liver fibrosis have several disadvantages including their complexity, the relatively long turn-around time, and high associated costs, and relatively low sensitivity. The study aims to test the feasibility of diagnosing and staging liver fibrosis via a flexible multi-analyte profiling (xMAP) technology by quantitative estimation of serological markers. Methods: Serum samples were collected from 81 patients and 14 healthy volunteers. Liver biopsy specimens were obtained from 23 patients for staging of liver fibrosis. Absolute quantification of transforming growth factor-β1 (TGF-β1), tissue inhibitor of matrix metalloproteinases (TIMP-1), laminin and collagen IV were obtained from each serum sample via ELISA and xMAP methods. Results: xMAP method had a higher efficacy than ELISA in disting uishing patients with stage IV liver fibrosis from healthy persons. Besides, xMAP could distinguish early stage liver fibrosis from late stage fibrosis, by demonstrating differences with respect to all the four biomarkers. Moreover, the receiver operating characteristics curves suggested discriminatory power of all four biomarkers on the xMAP platform, and that TGF-β1 had the best *P*-value. Conclusion: We demonstrated the efficacy of bead assay xMAP method in diagnosing and staging of liver fibrosis. xMAP method is a promising diagnostic tool for future clinical practice.

Keywords: Liver fibrosis, serologic markers, xMAP assay

Introduction

Liver fibrosis is defined as excessive accumulation of extracellular matrix proteins that occurs in most chronic liver disorders [1]. The onset of liver fibrosis is usually unidentifiable as the associated morbidity and mortality occurs only after onset of cirrhotic changes in the liver [2]. Cirrhotic changes in hepatic fibrosis usually manifest clinically as ascites, renal failure, hepatic encephalopathy, and variceal bleeding. Patients with cirrhosis are at a heightened risk of hepatocellular carcinoma [3]. The cirrhotic changes in liver are considered as irreversible, while the liver fibrosis (including fibrosis in advanced stages) has been recently shown to be reversible if the underlying cause of fibrosis is successfully treated [3-10]. With the advancement in the available treatment options for liver fibrosis, early diagnosis and accurate assessment of the stage of fibrosis could be a critical factor for successful treatment of hepatic fibrosis [11].

For the past five decades, liver biopsy has been the gold standard for assessment of inflammation and staging of hepatic fibrosis [3, 11, 12]. However, several recent studies have shown critical limitations of liver biopsy, including likelihood of sampling errors and lack of generalizability of results owing to the relatively small sample sizes. Moreover, inter- and intra-observer variability in the interpretation of the histopathological findings, high cost, and a small but significant risk of iatrogenic injury due to the invasive nature of the procedure [13-16]. Because of these limitations, liver biopsy is not suitable for use as a screening tool in high-risk individuals, or for making an accurate assessment of the fibrosis in certain patients [11].

Table 1. Baseline characteristics of study population

Characteristic	Number
Liver fibrosis/Healthy	81/24
Male/Female	59/46
Mean age, years	62.1±13.6
Etiology of liver fibrosis (n=81)	
Hepatitis B	50 (62)
Hepatitis C	6 (7)
Alcohol	11 (14)
Alcohol + Hepatitis B	1 (1)
Schistosome	4 (5)
Autoimmune	3 (4)
Cryptogenic	5 (6)
Fatty liver	1 (1)
Child-Pugh of liver cirrhosis (n=58)	
Class A	10 (17)
Class B	20 (35)
Class C	28 (48)
Comorbidities of liver cirrhosis (n=58)	
Upper GI bleeding	14 (24)
Ascites	26 (45)
Splenomegaly	50 (86)
Hepatic encephalopathy	5 (9)
Hepatorenal syndrome	4 (7)
Secondary infection	20 (35)

Values are n (%).

Alternative non-invasive methods for diagnosing and staging of liver fibrosis are increasingly being tested since the last decade, to help overcome these inherent limitations of liver biopsy [11, 12]. Currently available non-invasive methods range from advanced imaging studies to serum assay of various biomarkers [11, 12]. Existing imaging techniques, including transient elastography, acoustic radiation force impulse, and magnetic resonance imaging, allow for distinguishing between conditions with different pathological lesions, and in some cases help with identification of the nature of injury [11].

The interest in identifying and testing serological markers that are capable of assisting in the diagnosis liver fibrosis, has been on the rise in the recent years [11]. Estimation of serum markers is a non-invasive investigation, carries no risk of complications, has a negligible tendency for sampling errors and inter-observer variability, making it a particularly useful tool for dynamic monitoring of fibrogenesis. Moreover, the serological results can be a useful complement to the imaging techniques [11]. Many biomarkers have shown correlation with liver fibrosis, including procollagen type I carboxy terminal peptide, procollagen type III amino-terminal peptide, metalloproteinases, tissue inhibitors of matrix metalloproteinases (TIMPs), transforming growth factor-β1 (TGF-β1), hyaluronic acid, YKL-40 (chondrex), laminin, connective tissue growth factor, paraoxonase 1, and microfibril-associated glycoprotein 4 [11].

After extensive research it is now acknowledged that no single ideal serological marker exists, and that multiple markers showed be used concomitantly to help arrive at a definitive diagnosis [11, 12]. However, traditional diagnostic methods based on combined serological biomarkers usually involve collection of larger serum samples, have a longer turnaround time, are costly and involve complex procedures, thereby limiting their practical utility. Although the feasibility of a multiplex-approach involving multiple serological markers has been recently described, such an approach is likely to be hard to implement [11]. Applying specific antibodies to detect known serological markers in a flexible and straight forward multi-analyte profiling technology (xMAP), may be a step towards the multiplex-approach. The xMAP technology confers several advantages, such as reduced total assay time, fewer number of steps, and requirement for smaller sample volumes, compared to conventional enzyme-linked immunosorbent assay (ELISA) [17]. The present study is intended to serve as a pilot-test for assessing the feasibility of diagnosing and staging liver fibrosis via a Luminex 200 platform, targeting four direct serum markers (TGF-β1, TIMP-1, Laminin and Collagen IV).

Methods

Patient profile

A total of 81 patients with liver fibrosis, and 24 healthy volunteers were enrolled in the study. The baseline characteristics of study population were shown in **Table 1**. The study was approved by the Tongji Hospital Institutional Ethics Committee. All participants gave written informed consent. Blood samples were kept for 30 minutes to allow for clot formation. The

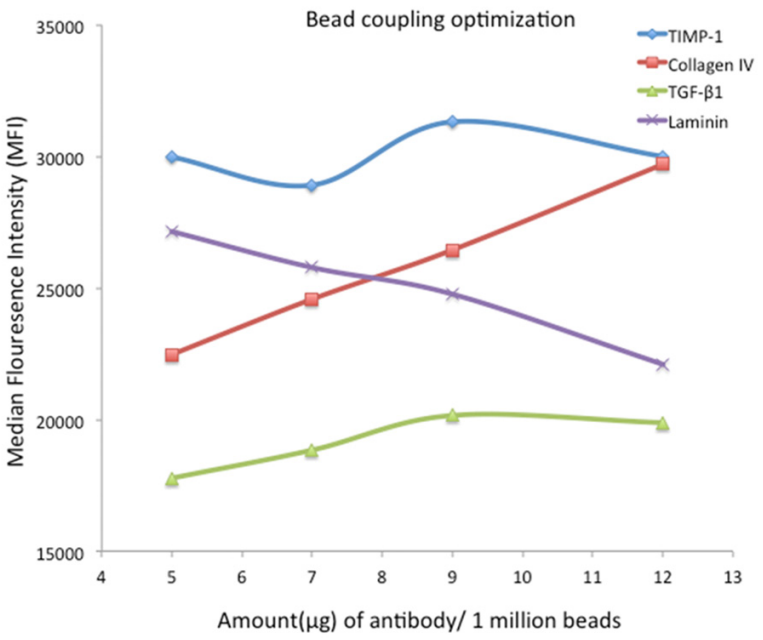

Figure 1. Microsphere coupling optimization reactions. 5 μg of antibody per 1 million beads served as the starting point with 3 higher concentrations (7, 9, 12 μg) also included to determine the optimal microsphere coupling reaction for each biomarker antibody.

serum samples were centrifuged and frozen at -80°C until their assay. Out of 81 patients, 58 were diagnosed as having stage IV liver fibrosis based on symptoms, ultrasound or CT scan findings. In the remaining 23 patients, liver fibrosis was identified by histopathological examination of liver biopsy specimen after staining with Masson's trichrome staining method. The staining procedure involved the following steps (in that order): Hematoxylin staining (10 min), xylidineponceau and acid fuchsin staining (10 min), phosphomolybdic acid staining (5 min), and aniline blue (10 min) staining. The trichrome staining showed collagenous fibrous tissue in blue color, cytoplasm of hepatocytes as red, and nuclei as black. Staging of specimen was performed according to the Knodell scoring system by two independent histopathologists.

ELISA

Serum samples were submitted to Wuhan Boster Biological Technology (Wuhan, China) for conducting ELISA for TGF-β1 (EK0513), TIMP-1 (EK0520), and laminin (EK0434) levels. For conducting ELISA for collagen IV level (F5704), serum samples were submitted to Shanghai Sinovac Biotech Ltd. (Shanghai, China).

Flexible multi-analyte profiling (xMAP)

Antibodies specific to each of the four serological markers were purchased: TIMP-1 (R&D, MAB970), TGF-β1 (R&D, MAB2461), laminin (Chemicon, LV1473020), and collagen IV (Chemicon, AB8201). The coupling efficiency of all antibody-conjugated Luminex microspheres was optimized. 20 μL of the serum sample was diluted with 80 μL of human serum sample diluents. Optimal amount of antibody-conjugated beads (9 μg for TGF-β1, 5 μg for laminin, 12 μg for collagen IV, 9 μg for TIMP-1) were incubated with diluted standard or sample in each well of a 96-well filter plate at room temperature for 30 minute on horizontal orbital microplates shaker at 300 rpm. After washing with PBS, 50 μL (10 μg/mL) of biotinylated goat anti-mouse secondary antibody (Oncogene, 0S02B-200 μg) was added to each well and incubated for 30 minutes on a shaker at 300 rpm. After re-washing in PBS, 50 μL of streptavidin-phycoerythrin (Sigma, B-4501) was added to each well and incubated for 10 min on shaker. Final step involved addition of 125 μL of assay buffer to each well and all fluorescent measurements were recorded with Luminex 200 (Luminex, USA).

Statistical analysis

All statistical analyses were conducted with Prism 6 Software (GraphPad, USA). Student's *t*-Test (2-tailed, 2-sample unequal variance) was performed. Receiver operating characteristics curve (ROC) analysis was conducted at 95% confidence interval (CI). All results are expressed as fractions. $P<0.05$ was considered to be statistically significant.

Results

Optimization of bead coupling

To maximize the performance of Luminex xMAP assay, the microsphere coupling reactions were

Table 2. Detection of TIMP-1, Collagen IV, TGF-β1, Laminin by ELISA and xMAP

	TIMP-1		Collagen IV		TGF-β1		Laminin	
	ELISA	xMAP	ELISA	xMAP	ELISA	xMAP	ELISA	xMAP
Stage IV Fibrosis (n=58)	314.6±134.6	6.9±3.7	17.9±17.5	22.6±63.9	137.8±89.4	1.3±0.7	107.3±92.2	49.6±61.7
Normal (n=14)	113.6±78.9	5.9±2.3	15.2±7.3	1.9±1.8	65.2±26.6	0.4±0.2	88.4±35.7	24.8±13.0
P-value	<0.001	0.21	0.38	0.02	<0.001	<0.001	0.22	0.006

TGF-β1, Transforming growth factor-β1; TIMP-1, Tissue inhibitor of matrix metalloproteinases.

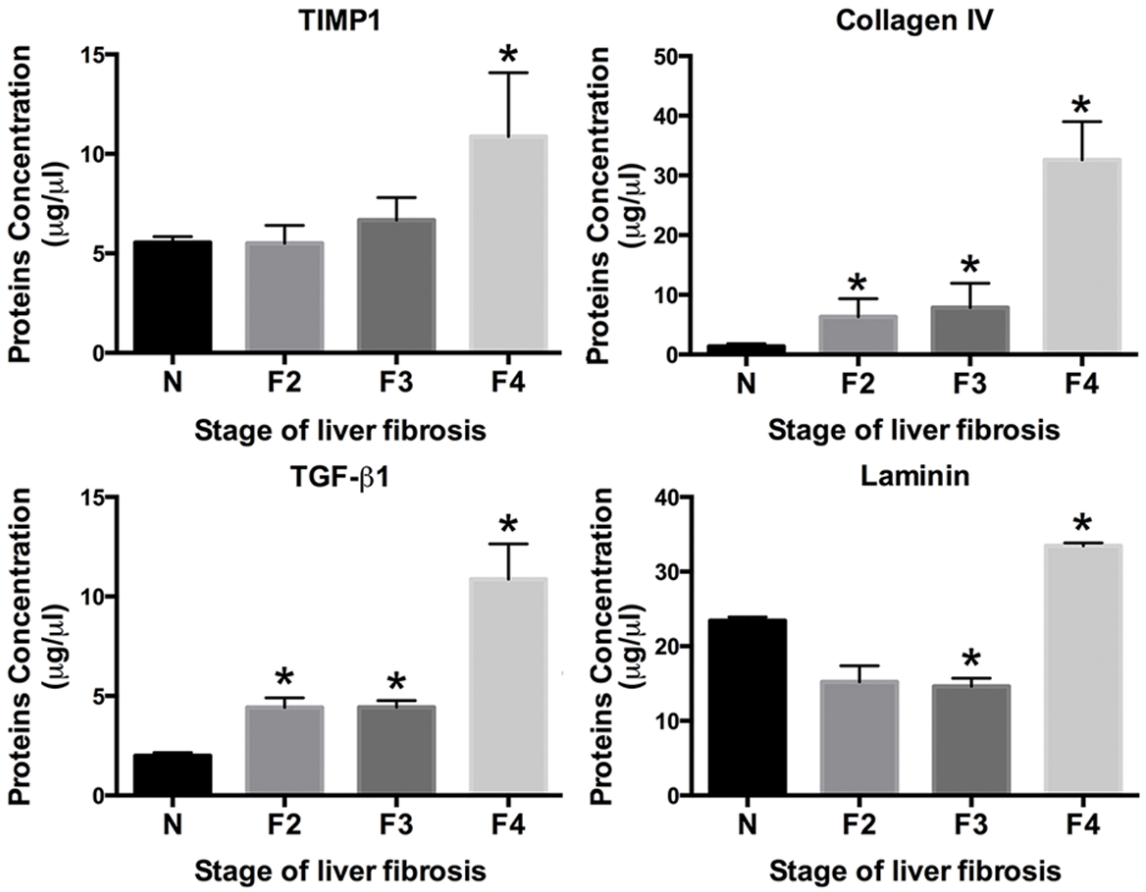

Figure 2. LuminexxMAP measurements of four serological markers. In normal participants (N; n=10), Stage II liver fibrosis (F2; n=11), Stage III liver fibrosis (F3; n=10) and Stage IV liver fibrosis (F4; n=2). *P<0.05.

optimized for all four target antibodies. Starting with the manufacturer's recommended starting point, four concentrations (5, 7, 9, 12 μg/L million beads) of antibodies were coupled to the microspheres (**Figure 1**). Concentration of antibodies that resulted in the highest median fluorescence intensity was used in the study: 9 μg for TGF-β1, 5 μg for laminin, 12 μg for collagen IV, and 9 μg for TIMP-1.

xMAP v.s. ELISA

To compare the performance of Luminex xMAP technology with conventional method, all four biomarkers were measured using xMAP and ELISA. A total of 58 serum samples from patients with advanced stage liver fibrosis, and 14 corresponding healthy volunteers were analyzed. The total protein concentration was found equivalent in all samples. ELISA tests showed 3-fold increase in TIMP-1 (P<0.001) and 2-fold increase in TGF-β1 (P<0.001) in liver fibrosis patients, as compared to healthy individuals; while there were negligible differences in collagen IV and laminin levels. In contrast, xMAP results indicated a 12-fold increase in collagen IV (P=0.02), 3-fold increase in TGF-β1 (P<0.001), and a 2-fold increase in laminin

Table 3. Staging of liver fibrosis based on TIMP-1, Collagen IV, TGF-β1, and Laminin measured by Luminex xMAP

Biopsy-based staging	TIMP-1	Collagen IV	TGF-β1	Laminin
F4 (n=2) *vs.* F3 (n=10)	+	+	-	+
F4 (n=2) *vs.* F2 (n=11)	+	+	+	+
F3 (n=10) *vs.* F2 (n=11)	-	-	-	-

'+' indicates power to discriminate between the two stages. TGF-β1, Transforming growth factor-β1; TIMP-1, Tissue inhibitor of matrix metalloproteinases.

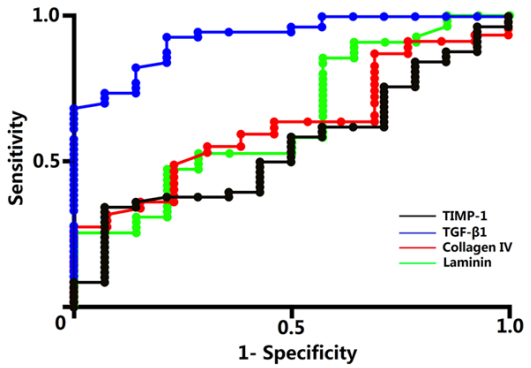

Figure 3. Receiver operating characteristic curves for serological markers todistinguish normal cases from Stage IV liver fibrosis. The area under curve values for TIMP-1, collagen IV, TGF-β1, and laminin were 0.55, 0.68, 0.93 and 0.67, respectively.

(*P*=0.006) in serum samples from patients with liver fibrosis, as compared to that of healthy patients, while showing negligible difference in TIMP-1 levels (**Table 2**).

xMAP v.s. liver biopsy

To test the feasibility of applying xMAP technology for staging liver fibrosis, additional serum and liver biopsy samples were taken from 23 liver fibrosis patients, and 10 matching serum samples from normal healthy participants. All 23 cases were staged according to the histopathological examination of biopsy specimen based on the Knodell scores. 11 patients were determined as liver fibrosis Stage II (F2), 10 cases were in Stage III (F3), and 2 cases were in Stage IV (F4). There were no cases of Stage I liver fibrosis. The levels of TIMP-1, collagen IV, TGF-β1, and laminin were measured in all serum samples via Luminex xMAP method. All four biomarkers showed statistically significant difference in their levels between F4 and normal samples (**Figure 2**). The levels of all serological markers were also significantly different between F2 and F4 serum samples. And three of the markers were able to distinguish F3 stage from F4 stage. However, none of the markers were able to differentiate F2 from F3 cases (**Table 3**).

ROC curves

ROC analysis was conducted to distinguish normal cases (n=14) from F4 cases (n=58). The area under curve (AUC) was determined for each of the four biomarkers and its value was used to distinguish non-predictive (AUC<0.5), less predictive (0.5<AUC<0.7), moderately predictive (0.7<AUC<0.9), and highly predictive (0.9<AUC<1) indicators. AUC values for TIMP-1, collagen IV, TGF-β1, and laminin were 0.55, 0.68, 0.93, and 0.67, respectively, indicating a potential role of TGF-β1 (*P*<0.001) as compared to less predictive power of other markers; TIMP-1 (*P*=0.55), collagen IV (*P*<0.05), and laminin (*P*=0.055) (**Figure 3**).

Discussion

Serological-biomarker based tests for diagnosing liver fibrosis have several inherent advantages, including their non-invasive nature, minimal susceptibility to inter-observer variability, and suitability for ongoing dynamic monitoring of liver fibrosis in comparison to liver biopsy [11, 12]. Owing to these advantages, the last decade has witnessed concerted efforts towards identifying serological biomarkers of liver fibrosis [11, 12]. The ideal characteristics of any serological biomarker of hepatic fibrosis would be a high level of sensitivity and specificity for staging liver fibrosis, low cost implications, reproducibility, and minimal incidence of false positive results [12]. A wide range of biomarkers of fibrosis have been discovered, but currently no single ideal marker is available [11]. While the identification of a single ideal biomarker would require further research, the key properties of currently identified biomarkers may be enhanced by superior diagnostic platforms.

In this study, we tested the feasibility of four direct serological markers on the Luminex xMAP plat form for diagnosis and staging liver fibrosis. The use of specific antibodies in xMAP technology has a number of advantages over the traditional methods (e.g. ELISA), including

increased sensitivity. The xMAP technology has recently been used for detection of low abundance serum cytokines in liver fibrosis patients [17-19]. Such enhanced sensitivity has indeed been the hallmark of the new biomarkers; however, efforts should also be directed towards enhancing the properties of previously identified biomarkers. In the present study, xMAP method demonstrated its ability to distinguish between normal and F4 serum samples in comparison to ELISA. For three out of the four markers (collagen IV, TGF-β1, and laminin) xMAP method measured statistically significant differences, while ELISA demonstrated significant difference in only two markers. All four markers of the xMAP platform were able to distinguish early stage (F2) from late stage (F4) liver fibrosis, with three of the markers also able to distinguish F3 from F4 stage. Moreover, ROC curves demonstrated the discriminatory power for all four markers on the xMAP platform, of which TGF-β1 had the highest level of significance.

As noted in **Table 1**, the concentration range measured by our xMAP method for all four serological markers was not consistent with ELISA results. Such discrepancies in the concentration range of the same markers between the two methods has been previously reported [20]. Based on a previous analysis, a constant conversion factor does not appear to exist between the two methods, and specific reference values must be established for each analysis [20, 21]. Indeed, our results can potentially serve as reference values for future xMAP studies of TIMP-1, collagen IV, TGF-β1, and laminin as targets.

While the absolute concentration range used with xMAP were different from ELISA, our xMAP measurements for the four serological markers were generally in agreement with the previous findings that showed TIMP-1 as controlling the activity of most metalloproteinases, and being positively correlated with the degree of liver fibrosis (r=0.73) [22, 23]. Our results demonstrated a similar up regulation of TIMP-1 in F4 serum as compared to normal serum. Similarly, collagen IV and laminin that are key extracellular matrix components have previously been shown to be unregulated with the advancement in stage of fibrosis [24-26]. In our study, their levels increased with increase in stage of fibrosis in comparison to their levels in normal serum. Also, the measurements of TGF-β1 in our study appeared same as previously reported trend of consistent up regulation of TGF-β1, a central mediator of fibrogenesis, in patients with fibrosis [27].

The slight-to-moderate discriminatory power (AUCs) of the four serological markers in this study appears to be due to the considerable variability in the measurements, and calls for further optimization of the protocol. Moreover, additional studies with larger sample size and including other indirect markers and antibodies are needed to confirm the diagnostic value of xMAP method. More specifically, future studies designed to investigate the extent of the benefit from concomitant use of direct serological biomarker testing and other diagnostic modalities (e.g. imaging and/or indirect markers). Such an approach would help facilitate the practical application of our findings in the diagnosis of liver fibrosis. In conclusion, our study demonstrates the potential diagnostic capability of xMAP method by measuring direct serological biomarkers for diagnosis and staging of liver fibrosis.

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Disclosure of conflict of interest

None.

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